Enhanced antitumor efficacy of an oncolytic adenovirus armed with an EGFR-targeted BiTE using menstrual blood-derived mesenchymal stem cells as carriers.

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Running title: mesenchymal stem cells as cell carrier for ICOVIR15-cBiTE

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ABSTRACT

Poor tumor targeting of oncolytic adenoviruses (OAdv) after systemic administration is considered a major limitation for virotherapy of disseminated cancers. The benefit of using mesenchymal stem cells as cell carriers for OAdv tumor targeting is currently evaluated not only in preclinical models but also in clinical trials. In this context, we have previously demonstrated the enhanced antitumor efficacy of OAdv-loaded menstrual blood-derived mesenchymal stem cells (MenSCs). However, although significant, the antitumor efficacy obtained was modest, and we hypothesized that a greater antitumor efficacy could be obtained arming the OAdv with a therapeutic transgene. Here we show that combining MenSCs with ICOVIR15-cBiTE, an OAdv expressing an epidermal growth factor receptor (EGFR)-targeting bispecific T-cell engager (cBiTE), enhances the antitumor efficacy compared to MenSCs loaded with the unarmed virus ICOVIR15. We found that MenSCs properly produce cBiTE after viral infection leading to a greater antitumor potency both *in vitro* and *in vivo*. These findings indicate the mutual benefit of combining MenSCs and armed OAdv and support the combination of ICOVIR15-cBiTE and MenSCs as a cancer treatment.

INTRODUCTION

Oncolytic adenoviruses (OAdv) have been extensively studied in clinical trials for the treatment of different cancer types, revealing good toxicological and safety profiles, but modest efficacy.[1] A key limitation upon OAdv systemic administration is virus biodistribution. Poor tumor targeting and limited tumor penetration contribute to such inefficacy.[2] The antiviral immune response also represents an important barrier for OAdv. Thus, the immune system can recognize the adenovirus in the bloodstream leading to its elimination. In particular, neutralizing antibodies raised after the first administration prevent the efficacy of subsequent doses. Moreover, virus replication within the tumor also triggers a potent antiviral response that limits ongoing infection of tumor cells, becoming dominant over tumor-specific immunity.[3]

The use of cell carriers to deliver oncolytic viruses to primary tumors and metastasis has been evaluated in order to overcome these obstacles. In this regard, mesenchymal stem cells (MSCs) have garnered interest as OAdv cell carriers given their natural tumor tropism and immunogenic properties.[4] We have recently reported the advantages of using menstrual blood-derived mesenchymal stem cells (MenSCs) as cell carriers for ICOVIR15, an OAdv developed in our laboratory.[5] We demonstrated not only an efficient OAdv tumor delivery, but also a significant, although modest, antitumor efficacy of OAdv-loaded MenSCs in combination with human peripheral blood mononuclear cells (hPBMCs). This increased antitumor effect was mainly mediated by monocyte activation leading to both T cell and natural killer cell activation.[5]

In a different line of research aimed at redirecting T cells towards tumor cells, we have recently engineered an armed OAdv (ICOVIR15K-cBiTE) expressing an epidermal growth factor receptor (EGFR)-targeting bispecific T-cell engager (cBiTE).[6] BiTEs are well-established immunotherapeutic molecules that combine the minimal binding domains (scFv) of two different

monoclonal antibodies fused by a short flexible linker.[7] One of the scFv recognizes the TCR CD3ε subunit whereas the second scFv targets tumor-associated antigens on cancer cells, leading to T-cell activation and target cell lysis. The major histocompatibility complex class I-independent and polyclonal mode of action of BiTEs,[8] offers the opportunity to redirect adenovirus-specific cytotoxic T lymphocytes to cancer cells. Our work revealed that ICOVIR15K-cBITE induces robust and specific-T cell activation and proliferation upon cancer cell infection, increasing the antitumor efficacy of the virus, both *in vitro* and *in vivo*.[6]

Combining these strategies, this study compares the antitumor efficacy of a cBiTE-armed (ICOVIR15-cBiTE) versus the unarmed (ICOVIR15) OAdvs in combination with MenSCs as cells carriers in the presence of hPBMCs. We show that MenSCs properly deliver both OAdvs to the tumor, allowing tumor viral replication and cBiTE production from the armed virus-infected cells. Enhanced antitumor efficacy is observed when the cBiTE-expressing OAdv is combined with MenSCs, demonstrating the potential benefit of using ICOVIR15-cBiTE-loaded MenSCs to improve the treatment outcome.

MATERIALS AND METHODS

Cell lines and viruses

The cancer cell lines A549 (human lung adenocarcinoma) and A431 (epidermoid carcinoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). A431-GL was generated by sorting A431 cells previously transduced with a lentiviral vector encoding GFP and luciferase. All tumor cell lines were maintained with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Thermo Fisher

Scientific, Waltham, MA, USA) at 37ºC, 5% CO_2. Cell lines were routinely tested for mycoplasma presence. Isolation and characterization of MenSCs has been previously described.[5] Experiments employing human PBMCs were approved by the ethics committees of the University Hospital of Bellvitge and the Blood and Tissue Bank (BST) from Catalonia. PBMCs of healthy donors were isolated from blood by ficoll (Rafer, Spain) density gradient centrifugation in Leucosep tubes (Greiner Bio-one, Kremsmünster, Austria) following the manufacturer's recommendations. T cells were isolated using the Rosette-Set Human T Cell Enrichment Cocktail (STEMCELL Technologies, Vancouver, Canada). For stimulation, T cells were cultured with CD3/CD28-activating Dynabeads (Thermo Fisher Scientific) at 1:3 bead-to cell ratio.

The oncolytic adenovirus ICOVIR15 have been previously described. [5] ICOVIR15-cBiTE was engineered to express cBiTE gene under the control of the viral major late promoter by homologous recombination in bacteria, similarly to previous described for ICOVIR15K-BiTE. [6]

Production of supernatants

A549 or MenSCs ($2.5x10^6$) were infected with ICOVIR15 or ICOVIR15-cBiTE (MOI=5 or 50 respectively) and supernatants were harvested 72 hours after infection. For binding assays, supernatants were concentrated 20x with Amicon Ultra-15 filter units with a molecular weight cut-off of 30 kDa (Merck Millipore, Darmstadt, Germany). Supernatants from uninfected cells were used as a negative mock control.

Binding assay

Target cells (A549 cells, $2x10^5$) were incubated with supernatants (generated from infected A549 cells) for 1 hour on ice. Cells were stained with the monoclonal M2 anti-FLAG antibody (Sigma Aldrich, St.Louis, MO, USA) as primary antibody and a goat anti-mouse IgG as secondary antibody (Thermo Fisher Scientific). Cells were analysed with a Gallios cytometer (Beckman

Coulter, Brea, Ca, USA). The FlowJo software (Tree Star, Ashland, OR, USA) was used for data analysis.

Virus cytotoxicity assays

Virus cytotoxicity assay were performed as previously described. [6] The inhibitory concentration 50 (IC_{50}) was calculated with GraphPad Prism v6.02 (GraphPad Software Inc.) by a dose-response nonlinear regression with a variable slope.

To assess the cBiTE-mediated cytotoxicity, A431-GL target cells ($3x10^4$) were co-cultured with $1.5x10^5$ T-cells (E:T=5) in 96-well plates. Co-cultures were mixed with 100µl of mock, ICOVIR15, or ICOVIR15-cBiTE supernatants (generated from MenSCs infected with the corresponding viruses). After 24h of incubation, cocultures were trypsinized and stained with LIVE/DEAD (Thermo Fisher Scientific). Viable GFP-expressing tumor cells were determined by flow cytometry (negative for LIVE/DEAD and positive for GFP). CountBright Absolute Counting Beads (Thermo Fisher Scientific) were used for absolute cell number determination. Cytotoxicity was expressed as the percentage of live cancer cells in co-cultures normalized to cancer cells cultured alone.

***In vivo* studies**

In vivo studies were performed at the ICO-IDIBELL animal facility (Barcelona, Spain) AAALAC unit 1155 and approved by IDIBELL's Ethical Committee for Animal Experimentation.

Lung adenocarcinoma xenograft tumors were established by implanting 5×10^6 A549 cells subcutaneously into both flanks of 8-week-old NOD scid gamma (NSG) mice. When tumors reached 100-120 mm^3, mice were randomized and distributed into groups. To evaluate systemic antitumor efficacy, $1x10^7$ human allogeneic PBMCs were administered to the mice by

intravenous injection. The next day, animals were treated with a single intraperitoneal dose of PBS, 1×10^{10} vp/mice of OAdv (ICOVIR15 or ICOVIR15-cBiTE) or MenSC/OAdv ($5x10^6$ cells previously infected with ICOVIR15 or ICOVIR15-cBiTE at MOI 50 for 24h).

Tumor volume was calculated according to the equation $V(mm^3) = \pi/6\times W^2\times L$, where W and L are the width and the length of the tumor, respectively. Data are expressed as the tumor size relative to the size at the beginning of the therapy (tumor growth). At the end of the study, animals were euthanized and tumors were collected. One half was frozen for DNA/RNA extraction, and the other half was fixed in 4% formaldehyde for 24h and embedded in paraffin.

DNA/RNA quantification by qPCR

Frozen tumor samples were disrupted using a mortar and pestle under liquid nitrogen. RNA and DNA were isolated with the DNA/RNA/protein kit (IBI Scientific, Dubuque, IA, USA) from approximately 25mg of homogenized tissue. RNA samples were treated with the TURBO DNA-free kit (Thermo Fisher Scientific) to remove traces of genomic DNA. RNA (1µg) was retrotranscribed with the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). Real-time analysis was performed in a LightCycler 480 Instrument II (Roche, Basel, Switzerland) in the presence of SYBR Green I Master (Roche). To quantify viral genomes and E1A/cBiTE transcripts in the tumor, 100ng of DNA and 40ng of cDNA were used, respectively. PCR conditions were: 95ºC 10 minutes, 40x cycles of 95ºC 15 seconds, 60ºC 1 minute and 72ºC 7 minutes. Viral genome primers were Ad18852: 5'-CTTCGATGATGCCGCAGTG-3' and Ad19047R: 5'-ATGAACCGCAGCGTCAAACG-3'. E1A primers were qE1AF: 5'-ATCGAAGAGGTACTGGCTGA-3' and qE1AR: 5'-CCTCCGGTGATAATGACAAG-3'; cBiTE primers were qBiTEF: 5'-CGGCGAGAAAGTGACAATGAC-3' and qBiTER: 5'-TTGGTGAGGTGCCACTTTTC-3' and b-actin primers were ACBTF: 5´-CTGGAACGGTGAAGGTGACA-3`and ACTBR 5´-GGGAGAGGACTGGGCCATT-3`.

Standard curves for viral genomes were prepared by serial dilutions of known copy numbers of a plasmid containing the adenovirus genome. β-actin expression was used to normalize E1a and cBiTE gene expression. In all cases, non-retrotranscribed RNA samples, equivalent to the amount cDNA loaded in the PCR, were run to discard genomic DNA contamination.

Histology and immunohistochemistry

A549 paraffin-embedded sections (5-µm thickness) of tumors sample were treated with an anti-Ad2/5E1A (SC-430, Santa Cruz Biotechnology) as primary antibody. Immunohistochemical staining was performed with EnVision (DAKO, Hamburg, Germany), according to manufacturers' instructions, and with hematoxylin. Images were acquired using the Nikon Eclipse 80i microscope running NIS elements BR software (Nikon Instruments Europe BV, Amsterdam, Netherlands).

Statistical analysis

Statistical comparisons between two groups were performed using the Mann-Whitney U test. For comparison of more than two groups, Kruskal-Wallis with Dunn *post-hoc* test was used. Statistical significance was established as $p<0.05$. Data are presented as the mean ±SD or SEM. All statistical analysis were calculated with GraphPad Prism software.

RESULTS

Generation and characterization of ICOVIR15-cBiTE generated from infected MenSCs.

The parental oncolytic adenovirus, ICOVIR15, has been previously described.[5] This virus is an E1a-Δ24-based oncolytic adenovirus with palindromic E2F binding sites in the *E1a* promoter and an insertion of Arg-Gly-Asp (RGD) in the HI-loop of the fiber knob that has shown a higher

MenSCs infection capability.[9] We engineered this virus to express an EGFR-targeting bispecific T-cell engager (cBiTE) under the control of the adenovirus major late promoter (Figure 1a).

To evaluate whether cBiTE insertion affected viral oncolytic properties, a dose-dependent cytotoxicity assay was performed. As shown in figure 1b, ICOVIR15-cBiTE retained oncolytic properties in vitro compared with its parental counterpart. We also compared virus production (both armed and parental virus) in MenSCs and A549 cell line (a highly permissive cell line commonly used for OAdv production). The total production of ICOVIR15 compared to ICOVIR15-cBiTE was similar for each cell line (mean of 4475 TU/cell vs 4185 TU/cell for ICOVIR15 and ICOVIR15-cBiTE on A549, and 2910 TU/cell vs 2475 TU/cell on MenSCs) indicating that cBiTE transgen insertion into ICOVIR15 genome does not affect total virus production (supplementary figure 1). Moreover, similar lower viral production was determined for both virus in MenSCs compared to A549 (1.5 and 1.6-fold decrease in production for ICOVIR15 and ICOVIR15-cBiTE, respectively, in MenSCs compared to A549), as previously described.[9]

We next determined whether the cBiTE encoded by ICOVIR15-cBiTE was properly secreted by MenSCs infected cells while retaining its EGFR-antigen binding property. For this purpose, flow cytometry–based binding assays were performed by using the FLAG tag fused to the cBiTE. Correct cBiTE binding to A549-EGFR$^+$ was detected only in the supernatants of ICOVIR15-cBiTE-infected MenSCs (figure 1c).

We next investigated the cBiTE-mediated cytotoxicity *in vitro*. To avoid the interference of virus-mediated cytotoxicity, we chose as a target the A431-GL EGFR$^+$ cell line, which is partly resistant to adenovirus infection because of its low coxsackievirus-adenovirus receptor expression. A431-GL cells were co-cultured with T-cells in the presence of supernatants obtained from non-infected (mock) or ICOVIR15/ICOVIR15-cBiTE-infected MenSCs. A

significant cytotoxicity was observed after 24h of incubation only in the presence of ICOVIR15-cBiTE supernatant (figure 1d). This result indicates that once cBiTE is expressed and secreted from infected cells, it can successfully lead to T-cell-mediated cytotoxicity of EGFR-expressing cells.

ICOVIR15-cBITE-loaded MenSCs enhances *in vivo* antitumor efficacy

We have recently reported the tumor homing properties and antitumor efficacy of ICOVIR15-loaded MenSCs after systemic administration in human tumor-bearing NSG mice in the presence of human PBMCs.[9] To determine whether cBiTE production from ICOVIR15-cBiTE infected MenSCs improved the previously described antitumor efficacy, NSG mice bearing subcutaneous A549 tumors were intravenously injected with human PBMCs and 24h later intraperitoneally treated with PBS (control group), ICOVIR15, ICOVIR15-cBiTE, MenSCs/ICOVIR15 (MenSCs previously infected with ICOVIR15 at MOI 50 for 24h), or MenSCs/ICOVIR15-cBiTE (MenSCs previously infected with ICOVIR15-cBiTE at MOI 50 for 24h). During the first week after treatment, all groups showed a tendency to control tumor growth compared to the control group, but differences were only statistically significant for the MenSCs/ICOVIR15-cBiTE group. Notably, from day 7 until the end of the experiment, although MenSCs/ICOVIR15 demonstrated improved tumor growth control compared to PBS and ICOVIR15 groups (1.2-fold decrease versus both groups), only the group treated with MenSCs/ICOVIR15-cBiTE showed a significant reduction in tumor growth compared to PBS (1.9-fold decrease) and to other treatment groups (1.9, 1.7, and 1.6-fold decrease versus ICOVIR15, ICOVIR15-cBiTE, and MenSCs/ICOVIR15, respectively) (figure 1e). Histology analysis of the tumors at the end of the experiment revealed the expression of the E1a protein in tumors of animals treated with the oncolytic adenovirus ICOVIR15 and ICOVIR15-cBiTE alone or in combination with MenSCs, confirming the correct delivery and amplification of both viruses in tumors (figure 1f).

Moreover, transcript analysis of collected tumors indicated correct viral transcripts (E1a) production in OAdv treated animals and, as expected, transgene transcript (cBiTE) generation only in ICOVIR15-cBiTE treated animals (figure 1g). However, when ICOVIR15-cBiTE was used, the amount of cBiTE transcripts was higher than the amount of E1a viral transcripts (figure 1g, white bars), and lower viral load (figure 1h) at the end of the experiment for ICOVIR15-cBiTE groups (alone or using MenSCs as carriers) were observed. These results point a competitive scenario between viral genes and cBiTE in terms of transcription or translation. However, this reduced amount of cBiTE-armed virus was not correlated with a reduced antitumor activity, probably due to cBiTE effect.

DISCUSSION

In 2016, we reported an initial clinical experience in the use of CELYVIR (autologous mesenchymal stem cells infected with ICOVIR5, a previous version of ICOVIR15, in children with advanced neuroblastoma (NCT01844661)).[10] Results from this clinical trial demonstrated good tolerance to the treatment and some clinical responses, including a complete remission. Over the last years, we have been trying to better understand the biology of MSCs after adenoviral infection and to develop an optimized CELYVIR version with enhanced antitumor properties and an easier production process. In previous work, we and others reported the advantages of using allogeneic MenSCs as cell carriers for OAdv tumor delivery.[5, 9, 11] Moreover, we pointed out the antitumor efficacy of MenSCs after OAdv infection in combination with human PBMCs. In this work we aimed at further improving the antitumor potential of our strategy by combining MenSCs with an EGFR BiTE-armed OAdv,[6] Although the advantages of expressing BiTEs from genetically modified mesenchymal stem cells have been previously demonstrated,[12] this is the first report on the benefit of the combination of MenSCs, BiTE immunotherapy and OAdv.

Our results prove proper cBiTE production from OAd-infected MenSCs, and an increased *in vitro* and *in vivo* cytotoxicity, confirming the existence of an improved tumor growth control by the cooperative action between OAd, cBiTE, and MenSCs. Of special interest for us is the *in vivo* antitumor efficacy comparison between the cBiTE-expressing versus the non-expressing OAdv in combination with MenSCs. Thus, the tumor growth difference between both groups is 7, 20, 2.3 and 1.6- fold decrease at day 4, 7, 11 and 14 post-treatment, respectively, being always in favor of the MenSCs/ICOVIR15-cBiTE group.

In summary, our results show that the combination of ICOVIR15-cBiTE and MenSCs could represent an attractive CELYVIR candidate for evaluation in clinical trials.

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CONFLICT OF INTEREST

The authors declare no conflict of interest exists.

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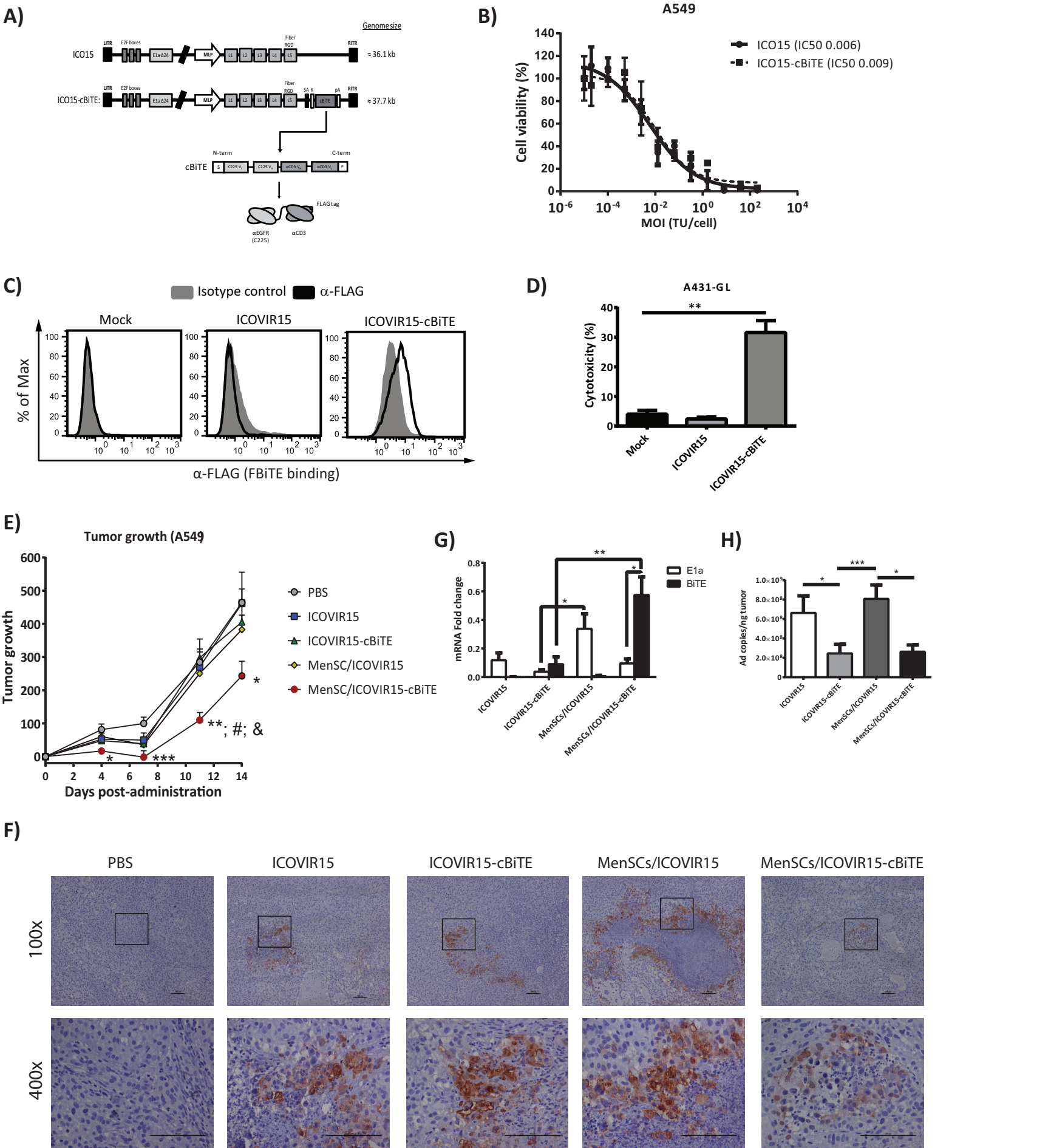
FIGURE LEGENDS

Figure 1. (a) Schematic representation of the genome of the viruses used in the study. L/RITR, left/right inverted terminal repeats; SA, splicing acceptor; K, kozac sequence; pA, polyadenylation signal; S, signal peptide; F, flag tag. (b) Cytotoxicity assay of ICOVIR15 versus ICOVIR15-cBiTE. A549 cells were incubated with serial dilutions of each OAdv, from 200 to 0 MOI. Cell viability was measured after 6 days and IC50 was calculated. The mean ± SD of triplicates is shown. (c) c-BiTE detection in supernatants from ICOVIR15 and ICOVIR15-cBiTE infected A549. A459 cells were incubated with concentrated supernatants from uninfected (Mock), ICOVIR15 (5 MOI) or ICOVIR15-cBiTE (5 MOI) infected cells and cBiTE binding was detected by flow cytometry. (d) cBiTE-mediated cytotoxicity in vitro. T-cells were co-cultured with A431-GL (target cells) in the presence of the different supernatants (from ICOVIR15 and ICOVIR15-cBiTE infected MenSCs). Twenty-four hours after co-culture the percentage of cytotoxicity of target cells (GFP+L/D-) was assessed by flow cytometry. Bars, mean ± SD of triplicates. **, $p<0.01$ by Kruskal-Wallis with Dunn post-hoc test (e) Antitumor efficacy of OAdv-loaded MenSCs *in vivo*. NSG mice bearing subcutaneous A549 tumors received an intravenous injection of human allogeneic PBMCs. Next day, mice were intraperitoneally injected with PBS, ICOVIR15, ICOVIR15-cBiTE, MenSCs previously infected

with ICOVIR15 or MenSCs previously infected with ICOVIR15-cBiTE. Tumor volume was monitored every 3-4 days. The mean of tumor growth ± SEM is shown. *, P< 0.05, ***, P< 0.001 MenSCs/ICOVIR15-cBiTE versus PBS group; #, P< 0.05 MenSCs/ICOVIR15-cBiTE versus ICOVIR15-cBiTE group; &, P< 0.05 MenSCs/ICOVIR15-cBiTE versus MenSCs/ICOVIR15 group by Kruskal-Wallis with Dunn post-hoc test. (f) Immunohistochemical staining of E1A of a representative tumor from each group is shown (scale bar 100μm). (g) cBiTE and E1A expression in tumors at the end of the experiment was determined by relative qRT-PCR using B-actin expression to normalize gene expression. Bars, mean ± SD of triplicates. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ by Kruskal-Wallis with Dunn post-hoc test (between different treatment groups) or by Mann Whitney U test (within each treatment group). (h) The presence of OAdv genomes in tumors at the end of the experiment was assessed by absolute qRT-PCR. Bars, mean ± SD of triplicates. ** $p<0.01$ by Kruskal-Wallis with Dunn post-hoc test.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αCD3 V_H
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FLAG tag
αEGFR (C225)
αCD3
B)
A549
ICO15 (IC50 0.006)
ICO15-cBiTE (IC50 0.009)
Cell viability (%)
MOI (TU/cell)
C)
Isotype control
α-FLAG
Mock
ICOVIR15
ICOVIR15-cBiTE
% of Max
α-FLAG (FBiTE binding)
D)
A431-GL
Cytotoxicity (%)
Mock
ICOVIR15
ICOVIR15-cBiTE
E)
Tumor growth (A549)
Tumor growth
Days post-administration
PBS
ICOVIR15
ICOVIR15-cBiTE
MenSC/ICOVIR15
MenSC/ICOVIR15-cBiTE
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